# Syntheses of Photoactive Analogues of Adenosine Diphosphate (Hydroxymethyl)pyrrolidinediol and Photoaffinity Labeling of Poly(ADP-ribose) Glycohydrolase[†]

Sushma Ramsinghani,[‡] David W. Koh,[§] Jean-Christophe Amé,[§] Mark Strohm,[§] Myron K. Jacobson,[§] and James T. Slama*,[‡]

*Department of Medicinal and Biological Chemistry, College of Pharmacy, University of Toledo, Toledo, Ohio 43606, and Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536*

*Received December 11, 1997; Revised Manuscript Received April 2, 1998*

ABSTRACT: Two isomeric azidoadenosyl analogues of adenosine diphosphate (hydroxymethyl)pyrrolidinediol [ADP-HPD; Slama, J. T., et al. (1995) *J. Med. Chem. 38*, 389−393] were synthesized as photoaffinity labels for poly(ADP-ribose) glycohydrolase. 8-Azidoadenosine diphosphate (hydroxymethyl)-pyrrolidinediol (8-$N_3$-ADP-HPD) inhibited the enzyme activity by 50% at ca. 1 $\mu$M, a concentration 80-fold lower than that where the isomeric 2-azidoadenosine diphosphate (hydroxymethyl)pyrrolidinediol did. [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD was therefore synthesized and used to photoderivatize poly(ADP-ribose) glycohydrolase. Irradiation of recombinant poly(ADP-ribose) glycohydrolase and low concentrations of [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD with short-wave UV light resulted in the covalent incorporation of the photoprobe into the protein, as demonstrated by gel electrophoresis followed by autoradiography or acid precipitation of the protein followed by scintillation counting. No photoincorporation occurred in the absence of UV light. The photoincorporation saturated at low concentrations of the photoprobe and photoprotection was observed in the presence of low concentrations of ADP-HPD, an indication of the specificity of the photoinsertion reaction. These results demonstrate that [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD can be used to specifically covalently photoderivatize the enzyme to characterize the polypetides that constitute the ADP-HPD binding site of poly(ADP-ribose) glycohydrolase. The photoincorporation reaction was further used to determine the ability of ADP-ribose polymers of varying size to compete with [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD for binding to the enzyme. Photoincorporation of [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD was inhibited by 80% in the presence of low concentrations of short, unbranched ADP-ribose oligomers (5−15 ADP-ribose units in length). No similar photoprotection was afforded by the addition of a high-molecular weight highly branched polymer. These results indicate that the photolabel shares a binding site with the short, linear polymer, but not with the long, highly branched polymer.

Poly(ADP-ribose) is an unusual polynucleic acid which is produced in the nuclei of most eukaryotic cells in response to DNA damage (*1*, *2*). Poly(ADP-ribose) is a homopolymer of adenosine diphosphate ribose units linked glycosidically through 1″ → 2′ ribosyl−adenosine bonds (*3*). Branching occurs through attachment of ADP-ribose residues via the 1‴ → 2″ ribosyl−adenosine bond (*4*). The size of the polymer varies from short unbranched oligomers to high-molecular weight polymers containing 200−400 ADP-ribose residues and possessing multiple branch points. The polymer is localized to the cell nucleus, where it is covalently attached to chromatin-associated proteins or histones.

ADP-ribose polymers are metabolized by the action of three enzymes: poly(ADP-ribose) polymerase (PARP)[1], poly(ADP-ribose) glycohydrolase (PARG), and ADP-ribosyl protein lyase. Poly(ADP-ribose) polymerase utilizes NAD as a substrate and catalyzes the initiation, elongation, and branching of the polymer. Poly(ADP-ribose) glycohydrolase catalyzes polymer degradation to ADP-ribose through hydrolysis of the glycosidic ribosyl−ribose bond. Catabolism is completed by ADP-ribosyl protein lyase, the enzyme which removes the terminal ADP-ribose residue from the ADP-ribose protein conjugate.

[†] Supported by research grants from the Ohio Division of the American Cancer Society, from the deArce Memorial Endowment Fund to J.T.S., and from the National Institutes of Health (CA 43894) to M.K.J.

* Corresponding author: Department of Medicinal and Biological Chemistry, College of Pharmacy, University of Toledo, Toledo, OH 43606-3390. Telephone: (419) 530-1925. E-mail: jslama@uoft02.utoledo.edu.

[‡] University of Toledo.

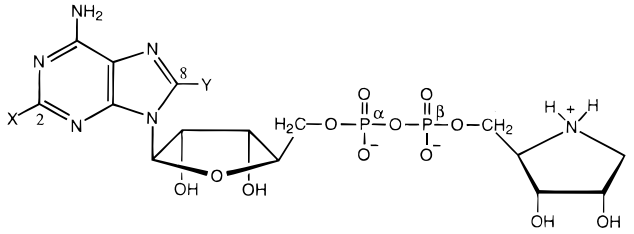
[§] University of Kentucky.

[1] Abbreviations: ADP-HPD, adenosine diphosphate (hydroxymethyl)pyrrolidine diol, **1**; DEAE, diethylaminoethyl substituent; DHB, dihydroxyboronyl-substituted; DMF, dimethylformamide; DTT, dithiothreitol; GST, glutathione *S*-transferase; HPD-P, (hydroxymethyl)-pyrrolidinediol 5′-phosphate, **5**; 2-$N_3$-ADP-HPD, 2-azidoadenosine diphosphate (hydroxymethyl)pyrrolidine diol, **2a**; 8-$N_3$-ADP-HPD, 8-azidoadenosine diphosphate (hydroxymethyl)pyrrolidine diol, **2b**; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(97)03038-9 CCC: $15.00 
Published on Web 05/06/1998

The metabolism of ADP-ribose polymers facilitates the recovery of dividing cells from DNA damage which would otherwise be lethal (*5*). Poly(ADP-ribose) synthesis is initiated only after DNA damage when PARP is activated through its binding to DNA strand breaks. Poly(ADP-ribose) may induce changes in chromatin structure, activate key regulatory enzymes or genes, facilitate DNA repair, or in some other way induce changes in nuclear metabolism favoring cell survival. ADP-ribose polymers exist only transiently in vivo, as they are rapidly degraded by PARG. Indirect evidence suggests that the regulation of PARG activity is important in the regulation of ADP-ribose polymer levels (*6*), although little is now known about the PARG mechanism or its regulation.

Adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD, **1**) is the first designed inhibitor that shows high



**1** : X = H, Y = H

**2a** : X = $N_3$, Y = H

**2b** : X = H, Y = $N_3$

potency and specificity for PARG (*7*). ADP-HPD additionally has the potential to serve as a tool for elucidating PARG structure and function through a photoaffinity approach.

Photoaffinity labeling has been extensively used to identify the ligand binding site of various enzymes and receptors (*8*). A photoaffinity label is designed by attaching a photoactive group (e.g., azido or diazirino) to a substrate, a ligand, or an inhibitor. After the probe is bound reversibly to the active site, the complex is irradiated with UV light. A photoreactive species is generated that chemically derivatizes the target through a rapid, irreversible, and covalent reaction with a binding site residue. A radiolabeled photoprobe facilitates the identification of the photoderivatized region of the enzyme.

In this study, we describe the synthesis of two photoactive azide analogues of the PARG inhibitor ADP-HPD: 2-azidoadenosine diphosphate (hydroxymethyl)pyrrolidinediol (2-$N_3$-ADP-HPD, **2a**) and 8-azidoadenosine diphosphate (hydroxymethyl)pyrrolidinediol (8-$N_3$-ADP-HPD, **2b**). One of these (**2b**) is further characterized and shown to be a potent and a specific photoaffinity label for poly(ADP-ribose) glycohydrolase.

## EXPERIMENTAL METHODS

*Materials.* Dimethylformamide (DMF) was stirred over activated molecular sieves (4 Å), distilled between 60 and 65 mmHg at 85 °C, and stored under nitrogen. Pyridine and methanol were dried over $CaH_2$ and distilled. 1,4-Dioxane was passed through a column of activity 1 alumina, stirred over $LiAlH_4$, and distilled. Diphenylphosphorochloridate and tributylamine were distilled under vacuum and stored under nitrogen. T4 polynucleotide kinase and 10× One-Phor-All buffer Plus were obtained from Pharmacia Biotech (Piscataway, NJ); nuclease P1 was obtained from Boeringher Mannheim (Indianapolis, IN), and [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Polyethyleneimine cellulose-coated plastic sheets with a short-wave fluorescent indicator for anion exchange TLC were purchased from Marcherey-Nagel (Düren, Germany).

*General Methods.* $^1$H NMR spectra were determined at 300 MHz at ambient probe temperature at a concentration of ca. 10 mg/0.5 mL. $^{13}$C NMR spectra were determined at 75 MHz. The chemical shifts are reported in parts per million and referenced to an internal standard of sodium 3-(trimethylsylyl)propionate-2,2,3,3-$d_4$ unless otherwise indicated. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrophotometer using KBr pellets formed by a hand-held press (Wilmad, Buena, NJ). Fast atom bombardment mass spectra were obtained at the University of Kentucky with a Concept 1H instrument (Kratos) at a weight resolution of 1500−2000 using glycerol as the matrix. HPLC was performed using a high-pressure gradient system and a UV detector operating at 254 nm. Analytical reversed phase HPLC was performed on a 3.9 × 300 mm reversed phase column (Waters, C-18 bondapak, 15−20 $\mu$m) using an ion-pairing technique. The solvents were (A) 20 mM $NaH_2PO_4$ and 2 mM tetrabutylammoniumdihydrogen phosphate (pH adjusted to 6.0 with 1 N NaOH) and (B) 50% (v/v) solvent A and 50% (v/v) acetonitrile. Chromatography was developed with the mobile phase consisting of 80% A and 20% B and changing linearly to 50% A and 50% B over the next 5 min and thereafter maintaining an isocratic flow for 25 min. The flow was maintained at 1.5 mL/min.

*(2R,3R,4S)-1-(Benzyloxycarbonyl)-2-[(phosphooxy)methyl]-pyrrolidine-3,4-diol (**4**).* Freshly distilled $POCl_3$ (0.71 mL, 7.6 mmol) was cooled in an ice bath, and water (68 $\mu$L, 3.8 mmol) followed by pyridine (0.6 mL, 7.6 mmol) was added while the mixture was being stirred. The reaction mixture solidified but liquified upon the addition of acetonitrile (0.85 mL). The starting alcohol **3** (*9*) (0.59 g, 1.9 mmol) was added as a solution in 0.85 mL of acetonitrile. The mixture was stirred at 4 °C for 2 h, at which time several small pieces of ice were added to destroy the excess $POCl_3$. Four milliliters of 1 M HCl was added and the reaction mixture stirred at ambient temperature for 45 min to remove the protecting group. The product was desalted on Amberchrome CG 71ms resin (1.5 × 45 cm) (a reversed phase type adsorbent) (TosoHaas, Philadelphia, PA). The column was developed with water. A large peak of pyridine preceded two smaller peaks. A test for inorganic phosphate showed that most of the inorganic phosphate had coeluted with pyridine. The latter two UV absorbing peaks were pooled, and the pH was adjusted to 7.5.

The monophosphate **4** was purified by anion exchange chromatography on a 1.5 × 43 cm column of DE-52 cellulose (Whatman, Maidstone, England), developed by the application of a linear gradient formed between 400 mL of 0.01 M $NH_4HCO_3$ (pH 7.5) and 400 mL of 0.2 M $NH_4HCO_3$ (pH 7.5). Fractions (7.5 mL) were collected, and absorbance at 254 nm was measured. A single major peak eluting about midway in the gradient was pooled and lyophilized. After several lyophilizations, **4** was obtained as a white amorphous solid (0.51 g, 77% yield). The structure of **4** was confirmed

by comparison to authentic material using TLC, HPLC, and $^1$H NMR (*9*).

*(2R,3R,4S)-2-[(Phosphooxy)methyl]pyrrolidine-3,4-diol (**5**).* The benzyloxycarbonyl phosphate ester **4** (0.27 g, 0.77 mmol) was dissolved in 10 mL of water and 30 mL of methanol and subjected to catalytic hydrogenation (50 psi, 5% Pd on carbon, 340 mg) for 4 h. TLC showed that by this time all of the starting material had been consumed. The catalyst was removed by filtering through a Celite filter aid. The solvent was evaporated under reduced pressure to obtain an oil in quantitative yield that was dissolved in water and lyophilized: TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f = 0.13$; $^1$H NMR ($D_2O$) $\delta$ 3.33−3.38 (d, 1H, $CH_2N$), 3.45−3.50 (dd, 1H, $CH_2N$), 3.68−3.73 (m, 1H, CHN), 3.97−4.06 (m, 1H, $CH_2OP$), 4.19−4.27 (m, 1H, $CH_2$-OP), 4.37−4.41 (m, 2H, 2CHOH); $^{13}$C NMR ($D_2O$) $\delta$ 52.20 (C-5), 62.95 and 63.01 (C-6), 64.02 and 64.08 (C-2), 72.59 and 73.86 (C-3 and C-4).

*(2R,3R,4S)-1-(tert-Butyloxycarbonyl)-2-[(phosphooxy)methyl]pyrrolidine-3,4-diol (**6**).* Lyophilized **5** (0.162 mg, 0.77 mmol) was dissolved in 5.8 mL of water and 3.9 mL of methanol. Triethylamine was added dropwise to adjust the pH of the solution to 10. Di-*tert*-butyl dicarbonate (0.336 g, 1.54 mmol) was added to the stirred solution of **5** as a solution in 13 mL of methanol. The flask was stoppered and the reaction mixture stirred overnight. TLC showed complete conversion to a higher-$R_f$ material. The solvent was removed in vacuo and the residue chased three times with a water/toluene mixture. The oily residue was dissolved in water and lyophilized twice to yield a colorless flaky solid (0.29 g, 91%): TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f = 0.25$.

Compound **6** was lyophilized and isolated as a ditriethylammonium salt. For the purpose of obtaining NMR and microanalysis, it was converted to the sodium form by cation exchange chromatography (AG 50W-X8, 50−100 mesh $Na^+$ form): $^1$H NMR ($D_2O$) $\delta$ 1.48 [s, 9H, $(CH_3)_3C$], 3.34−3.43 (m, 1H, $CH_2N$), 3.51−3.64 (m, 1H, $CH_2N$), 3.77 (broad s, 1H, CHN), 3.97−4.10 (m, 2H, $CH_2OP$), 4.31 (broad s, C*H*OH), 4.37−4.42 (dd, 1H, C*H*OH); $^{13}$C NMR ($D_2O$, $CD_3$-OD as an external reference with $\delta$ 49.0) $\delta$ 28.64 [$(CH_3)_3$], 51.03−51.56 (d, $CH_2N$), 63.22 ($CH_2OP$), 63.77−63.89 (CHN), 70.04−70.36 (d, CHOH), 73.57−74.05 (d, CHOH), 82.77−82.91 [$(CH_3)_3C$], 157.40 (OCO). Anal. Calcd for $C_{10}H_{19}O_8NP\cdot 2Na\cdot {}^1/_2H_2O$: C, 32.80; H, 5.23; N, 3.82. Found: C, 32.47; H, 5.52; N, 3.82.

*2-Azidoadenosine 5′-Monophosphate (**7a**).* Nucleotide **7a** was prepared from 2-chloroadenosine in three steps by first converting it into the hydrazino analogue which was treated with nitrous acid to produce 2-azidoadenosine (*10*). This nucleoside was selectively phosphorylated on the 5′-hydroxyl with $POCl_3$ in trimethyl phosphate (*11*) to obtain **7a**.

*(tert-Butyloxycarbonyl)-2-azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (t-Boc-2-azido-ADP-HPD, **8a**).* 2-Azidoadenosine 5′-monophosphate ($NH_4^+$ form) **7a** (135 mg, 0.35 mmol) was dissolved in 25 mL of anhydrous methanol, and trioctylamine (263 $\mu$L, 0.6 mmol) was added and the mixture stirred until a solution was obtained. The solvent was evaporated in vacuo and anhydrous DMF (3 mL) added to dissolve the residue and evaporated in vacuo. The addition and evaporation of DMF was repeated twice to obtain a white residue. The white residue was redissolved in anhydrous dioxane (9 mL), and diphenylphosphorochloridate (109 $\mu$L, 0.53 mmol) was added, followed immediately by tributylamine (125 $\mu$L, 0.53 mmol). A white precipitate formed, and the reaction mixture was stirred under nitrogen. TLC (silica gel, 5:2 ethanol/1 M $NH_4OAc$) after 3 h showed that most of the starting material had been consumed. The solvent was evaporated in vacuo, and ether (30 mL) was added to the oily residue to precipitate the product. The precipitate was allowed to stand at 0 °C for 30 min and the ether decanted. The residue was dried by the addition and evaporation of 3 mL of anhydrous dioxane (repeated three times) and then dissolved in 9 mL of anhydrous DMF. Phosphate ester **6** (217 mg, 0.53 mmol, ditriethylammonium form) was dissolved in 3 mL of anhydrous pyridine and the mixture added to the solution of activated 2-$N_3$-AMP. The reaction mixture was stirred overnight under nitrogen and protected from light. TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) showed a major spot different from the reactants. The product was isolated by precipitation with ether, and the residue was dissolved in water and diluted to 350 mL. The pH was adjusted to about 7.5 with 1 M $NH_4OH$; the sample was applied to a column of benzyl-DEAE-cellulose (1.5 × 100 cm), and the chromatography was developed by applying a linear gradient formed between 0.01 and 0.4 M $NH_4HCO_3$ (800 mL each, pH 7.5). Fractions (7.5 mL) were collected, and the absorbance was measured at 254 nm. A major peak eluting midway in the gradient was lyophilized. Reversed phase HPLC analysis of the lyophilized material showed peaks at 8.0, 11.2, and 13.7 min. The latter two peaks resulted from the tetrazole−azide equilibrium of the product; however, the peak at 8 min represented an impurity. This was removed by rechromatography on a preparative HPLC column (Waters C-18 bondapak, 125 Å, 15−25 $\mu$m, 19 × 300 mm) (flow rate of 20 mL/min, starting with 80% A and 20% B for 5 min, changing linearly to 50% A and 50% B, and maintaining this for 25 min). The peaks representing the tetrazole−azide tautomers were collected and lyophilized. The lyophilized material was analyzed on reversed phase HPLC to obtain two peaks (in equilibrium, retention times = 13.8 and 15.2 min); the UV absorption spectrum (pH 7.5) showed absorption maxima at 311.0, 273.5, and 231.0 nm: TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f = 0.54$; $^1$H NMR ($D_2O$; referenced to HDO at $\delta$ 4.67) $\delta$ 1.23 [s, 9H, $(CH_3)_3C$], 3.13−3.17 (m, 1H, $CH_2N$), 3.28−3.33 (m, 1H, $CH_2N$), 3.60 (broad s, 1H, CHN), 3.90 (broad s, 2H), 4.06 (broad s, 2H), 4.15−4.25 (m, 3H), 4.38 (broad s, 1H, CHOH of pyrrolidine), 4.66 (broad s, 1H, CHOH of pyrrolidine), 5.87−5.88 and 6.01−6.02 (two d, 1H, anomeric H), 8.3 and 8.45 (two broad s, 1H, adenosyl H).

*2-Azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (2-Azido-ADP-HPD, **2a**).* Lyophilized *t*-Boc-2-$N_3$-ADP-HPD (**8a**) was dissolved in 2 mL of trifluoroacetic acid and the mixture stirred for 45 min. Trifluoroacetic acid was evaporated in vacuo and chased twice with water. The residue was diluted with water (80 mL), with the pH adjusted to 7.5 with 1 M $NH_4OH$, and applied to a column of DE-52 cellulose (1.5 × 34 cm). Chromatography was developed by a linear gradient formed between 95 mL each of 0.01 and 0.4 M $NH_4HCO_3$ at pH 7.5. Fractions (7.5 mL) were collected, and a major peak of **2a** contaminated with inorganic phosphate eluted in the first half of the gradient.

The product peak was collected, lyophilized, and desalted by pH-dependent boronate affinity chromatography. DHB-Bio Rex 70 was prepared according to the procedure of Wielckens et al. (*12*). The lyophilized material was dissolved in 30 mL of 0.25 M $NH_4OAc$ and the pH adjusted to 8.8 with 1 M $NH_4OH$. The sample was applied to a column of DHB-Bio Rex 70 (1.0 × 25 cm), and the column was developed with about 150 mL of 0.25 M $NH_4OAc$ (pH 8.8) until all the inorganic phosphate eluted off the column. A major peak was next eluted with water. The fractions were pooled and lyophilized three times to completely remove ammonium acetate. 2-$N_3$-ADP-HPD was obtained as a white fluoculant solid (17 mg, yield of approximately 10%): TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f$ = 0.37, (silica gel, 5:2:3 *n*-butanol/concentrated acetic acid/water) $R_f$ = 0.37; HPLC (reversed phase, ion-pair) retention time = 3.3 (minor), 6.1 min (major) (tetrazole−azide equilibrium); UV absorption spectrum (pH 7.0) $\lambda_{max}$ = 310.0, 271.0, and 230.0 nm; $^1$H NMR ($D_2O$, referenced to HDO at $\delta$ 4.67), $\delta$ 3.15−3.22 (m, 1H, $CH_2N$), 3.27−3.36 (m, 1H, $CH_2N$), 3.58 (broad s, CHN), 3.98−4.09 (m, 3H), 4.15−4.22 (m, 4H), 4.36−4.37 (m, 1H, CHOH of pyrrolidine), 5.76−5.78 and 5.93−5.94 (two d, 1H, anomeric H), 8.07 and 8.33 (two s, 1H, adenosyl H); IR 2141 cm$^{-1}$ (moderate intensity, $N_3$ stretching); fast atom bombardment mass spectrum (negative ion) calcd for $(M - H)^-$ ($C_{15}H_{23}O_{12}N_9P_2$) *m*/*z* 582, found *m*/*z* 582 (base peak, relative intensity of 100%); fragment ion peak calcd for $[(M - H) - N_2 + 2H]^-$ *m*/*z* 556, found *m*/*z* 556 (relative intensity of 40%).

*8-Azidoadenosine 5′-Monophosphate (**7b**).* 8-Azidoadenosine (Aldrich, Milwaukee, WI) was phosphorylated on its 5′-hydroxyl with $POCl_3$ in trimethyl phosphate according to the procedure of Yoshikawa et al. (*11*).

*(tert-Butyloxycarbonyl)-8-azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (t-Boc-8-$N_3$-ADP-HPD, **8b**).* 8-Azidoadenosine 5′-monophosphate (85 mg, 0.22 mmol), **7b**, was dissolved in anhydrous methanol (15 mL), and trioctylamine (79 mg, 0.22 mmol) was added via a positive displacement pipet. The mixture was stirred for about 20 min until a solution was obtained. The solvents were removed in vacuo. Anhydrous DMF (1.5 mL) was added to dissolve the residue and then evaporated in vacuo. This was repeated twice. The oily residue was dissolved in 1.0 mL of anhydrous dioxane, and diphenylphosphorochloridate was added (68 $\mu$L, 0.33 mmol), followed immediately by tributylamine (105 $\mu$L, 0.44 mmol). The reaction mixture was stirred under nitrogen in the dark. TLC (silica gel, 5:2 ethanol/1 M $NH_4OAc$) after 3 h showed that almost all of the 8-$N_3$-AMP had been consumed. The solvent was evaporated in vacuo, and the product was isolated by precipitation with 6 mL of cold ether. After the mixture was cooled for 30 min at 0 °C, ether was decanted and the residue was dried three times by the addition and evaporation of anhydrous dioxane (1.5 mL). It was then dissolved in 1.0 mL of anhydrous DMF. The ditriethylammonium salt of **6** (137 mg, 0.33 mmol) was dissolved in 2.5 mL of anhydrous DMF and added to the solution of activated 8-azido-AMP, followed immediately by 8 mL of anhydrous pyridine. The reaction mixture was stirred overnight, under nitrogen and protected from light. The solvents were removed in vacuo, and ether (8 mL) was added to precipitate the product. After the solution stood for 30 min on ice, ether was decanted and the product was dissolved in water and purified by anion exchange chromatography on a column of benzyl-DEAE-cellulose (1.5 × 96 cm). The sample was diluted to 50 mL and the pH adjusted to 7.5 with 1 M $NH_4OH$. Chromatography was developed by applying a linear gradient of 400 mL each of 0.01 and 0.4 M $NH_4HCO_3$ at pH 7.5. Fractions (7.5 mL) were collected and monitored by UV absorbance (254 nm). The major peak eluting after about two-thirds of the gradient was pooled and lyophilized several times to yield a white fluocculent solid (75.3 mg, yield 50%): TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f$ = 0.59; HPLC (reversed phase, ion-pair) single peak at 14.3 min; UV absorption spectrum (pH 7.5) $\lambda_{max}$ = 281.0 nm; $^1$H NMR ($D_2O$, referenced to the HDO peak at $\delta$ 4.67) $\delta$ 1.21 [s, 9H, $(CH_3)_3C$], 3.10−3.15 (m, 1H, $CH_2N$), 3.24−3.30 (m, 1H, $CH_2N$), 3.51 (broad s, 1H, CHN), 3.88 (broad s, 2H), 4.02−4.16 (m, 5H), 4.38−4.41 (t, 1H), 4.48−4.92 (t, 1H), 5.78−5.79 (d, 1H, anomeric H), 7.99 (s, 1H, adenosyl H).

*8-Azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (8-Azido-ADP-HPD, **2b**).* *t*-Boc-8-$N_3$-ADP-HPD (**8b**, 28 mg, 0.04 mmol) was dissolved in 2 mL of trifluoroacetic acid and the mixture stirred at ambient temperature for 45 min. Trifluoroacetic acid was removed in vacuo and chased twice with water. The residue was diluted to 50 mL with water and the pH adjusted to 7.5 with 1 M $NH_4OH$. The sample was applied to a column of anion exchange resin AG 1-X2 (200−400 mesh, acetate form) and chromatography developed with a linear gradient formed between 400 mL each of water and 2 M acetic acid. A major peak which eluted in the second half of the gradient was collected and lyophilized several times to obtain a white flocculent solid: TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f$ = 0.27; reversed phase HPLC single peak at 2.4 min; UV absorption spectrum (pH 7.5) $\lambda_{max}$ = 281.0 nm; $^1$H NMR ($D_2O$, referenced to HDO at $\delta$ 4.67) $\delta$ 3.33−3.37 (d, 1H, $CH_2N$), 3.46−3.50 (d, 1H, $CH_2N$), 3.75 (broad s, 1H, CHN), 4.18−4.38 (m, 7H), 4.58−4.62 (t, 1H, CHOH), 5.04−5.08 (t, 1H, CHOH), 5.96−5.98 (d, 1H, anomeric H), 8.19 (s, 1H, adenosyl H); IR 2159 cm$^{-1}$ (strong intensity, $N_3$ stretching); fast atom bombardment mass spectrum (negative ion) calcd for $(M - H)^-$ ($C_{15}H_{23}O_{12}N_9P_2$) *m*/*z* 582, found *m*/*z* 582 (base peak, relative intensity of 40%); fragment ion peak calcd for $[(M - H) - N_2 + 2H]^-$ *m*/*z* 556, found *m*/*z* 556 (relative intensity of 100%).

*8-Azidoadenosine 3′-Monophosphate (**9**).* The synthetic procedure was adapted from that described for similar nucleotides (*13*−*15*). The sodium salt of 3′-AMP (52.05 mg, 0.15 mmol) was dissolved in 2 mL of 1 M sodium acetate buffer at pH 4.0 in a vial fitted with a tight fitting Teflon-lined cap. Bromine/water (2 mL) (10 $\mu$L of bromine dissolved in 2 mL of water) was added, the vial tightly stoppered, and the reaction allowed to proceed overnight. Excess bromine was removed by bubbling a stream of air through the mixture and the product purified by anion exchange chromatography on DE-52 cellulose (1.5 × 21 cm) by applying a linear gradient of 400 mL each of 10 to 250 mM $NH_4HCO_3$ at pH 7.5. 8-Br-3′-AMP had a characteristic $\lambda_{max}$ at 265 nm. The 8-Br-3′-AMP (36.2 mg, 0.085 mmol) was dried by dissolving it in anhydrous DMF and evaporating the DMF in vacuo. The residue was dissolved in 0.4 mL of anhydrous DMF, and sodium azide (28 mg, 0.43 mmol) was

added. The reaction mixture was stirred at 75 °C for 18 h. The solvent was removed in vacuo, and the product 8-$N_3$-3′-AMP was purified by anion exchange chromatography on DE-52 cellulose (1.5 × 44 cm) by applying a linear gradient of 400 mL each of 10 to 250 mM $NH_4HCO_3$ at pH 7.5. The 8-$N_3$-3′-AMP was obtained in 60% yield and had a characteristic $\lambda_{max}$ at 281 nm: IR 2152 $cm^{-1}$ (strong intensity, $N_3$ stretching).

*[$^{32}$P]-8-Azidoadenosine 5′-Monophosphate ([$^{32}$P]-8-$N_3$-5′-AMP, [$^{32}$P]**7b**)*. The synthesis of [$^{32}$P]-8-$N_3$-5′-AMP made use of T4 polynucleotide kinase and nuclease P1 (*16*). To an aqueous solution of 8-$N_3$-3′-AMP (0.8 mg in 200 μL, 2 μmol) in a 1.5 mL polypropylene Eppendorf centrifuge tube was added 2 mCi [γ-$^{32}$P]ATP [specific activity of 7000 Ci/mmol obtained as a solution in 12 μL of 200 mM Tris-HCl (pH 9.0) and 25 mM DTT] and 24 μL of 10× One-Phor-All Buffer Plus followed by 20 units of T4 polynucleotide kinase (ca. 4 μL) (Pharmacia). The reaction mixture was incubated at 37 °C in a shaking water bath for 4 h. Thereafter, ATP [1 mg dissolved in 20 μL of 50 mM Tris-HCl (pH 7.0)] and an additional 30 units of T4 polynucleotide kinase and 5 μL of 10× One-Phor-All Buffer Plus were added, and incubation was continued overnight. A TLC [polyethyleneimine cellulose-F, 0.75 M $KH_2PO_4$, pH adjusted to 3.5 with concentrated HCl] followed by autoradiography showed complete consumption of [γ-$^{32}$P]ATP and the formation of a major product ([$^{32}$P]-8-$N_3$-3′,5′-ADP) and a minor product ([$^{32}$P]-8-$NH_2$-3′,5′-ADP). Nuclease P1 (4 units) in 500 mM Tris-HCl (pH 9.0, 24 μL) was added to the reaction vial and incubation at room temperature continued overnight. A TLC on polyethyleneimine cellulose (0.75 M $KH_2PO_4$, pH 3.5) showed formation of [$^{32}$P]-8-$N_3$-5′-AMP as the major product and [$^{32}$P]-8-$NH_2$-5′-AMP as the minor product. The product was purified by anion exchange chromatography on a benzyl-DEAE-cellulose column ($HCO_3^-$, 1.5 × 23 cm) using a linear gradient formed between 300 mL each of 5 and 250 mM ammonium bicarbonate (pH 7.5). The product [$^{32}$P]-8-$N_3$-5′-AMP eluted in the second half of the gradient, followed by [$^{32}$P]-8-$NH_2$-5′-AMP. The fractions containing [$^{32}$P]-8-$N_3$-5′-AMP were pooled and lyophilized twice: TLC (polyethyleneimine cellulose, 0.75 M $KH_2PO_4$, pH 3.5) $R_f = 0.60$.

*[α-$^{32}$P]-1-(tert-Butyloxycarbonyl)-8-azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol ([α-$^{32}$P]-t-Boc-8-$N_3$-ADP-HPD, [$^{32}$P]**8b**)*. The protected pyrrolidine **6** (6.28 mg, 20 μmol) was dissolved in 0.8 mL of anhydrous DMF, and 16.2 mg of *N*,*N*′-carbonyldiimidazole (100 μmol) was added to it. The reaction mixture was stirred for 2 h under ambient conditions. TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) showed complete conversion of the starting material to a higher-$R_f$ material ($R_f = 0.71$). Anhydrous methanol (3.3 μL, 80 μmol) was added, and the reaction mixture was allowed to stand at room temperature for 30 min. The lyophilized [$^{32}$P]-8-$N_3$-5′-AMP ([$^{32}$P]**7b**) was dissolved in 0.4 mL of anhydrous methanol, and 4.6 μL (20 μmol) of anhydrous tributylamine was added. After the mixture was stirred for 30 min, a solution was obtained. The solvent was removed in vacuo. The residue was dried by addition and evaporation of 0.4 mL of anhydrous DMF. The residue was dissolved in 0.4 mL of anhydrous DMF and the solution of activated pyrrolidine **10** added to it. The reaction mixture was stirred in the dark under ambient conditions overnight. The solvent was removed in vacuo, and the product was purified by anion exchange chromatography using benzyl-DEAE-cellulose (bicarbonate form, 1.5 × 20 cm). Chromatography was developed by applying a linear gradient formed between 400 mL each of 0.01 and 0.25 M $NH_4HCO_3$ at pH 7.5, and fractions were collected. The radioactivity was measured by liquid scintillation counting. The fractions containing the product were pooled and lyophilized. Autoradiography of the TLC plate (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water) showed the presence of a small amount of [$^{32}$P]-8-$N_3$-5′-AMP ($R_f = 0.16$) and a major spot of [α-$^{32}$P]-*t*-Boc-8-$N_3$-ADP-HPD ($R_f = 0.49$).

*[α-$^{32}$P]-8-Azidoadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol ([α-$^{32}$P]-8-$N_3$-ADP-HPD, [$^{32}$P]**2b**)*. The *t*-Boc protected dinucleotide [$^{32}$P]**8b** was dissolved in 1.0 mL of trifluoroacetic acid and the mixture stirred magnetically for 45 min. The acid was removed in vacuo and chased twice with water. The residue was dissolved in 25 mL of 0.25 M ammonium acetate, and 1 drop of concentrated $NH_4OH$ was added to raise the pH to 8.8. The sample was applied to DHB-Bio Rex 70 (0.7 × 25 cm), and the chromatography was developed first by 300 mL of 0.25 M ammonium acetate at pH 8.8, followed by 50 mL of water. [α-$^{32}$P]-8-$N_3$-ADP-HPD eluted immediately into the water. The radioactive fractions were pooled and lyophilized several times to yield a white solid: TLC (6:3:1 2-propanol/concentrated $NH_4OH$/water) $R_f = 0.26$, (4.5:1.5:1:0.17:2.83 *n*-butanol/acetone/concentrated acetic acid/concentrated $NH_4OH$/water) $R_f = 0.28$. Autoradiography of the TLC plate demonstrated that the photolabel was free from any [$^{32}$P]-8-$N_3$-5′-AMP. The specific activity of [α-$^{32}$P]-8-$N_3$-ADP-HPD was determined to be 0.5−2.0 mCi/μmol.

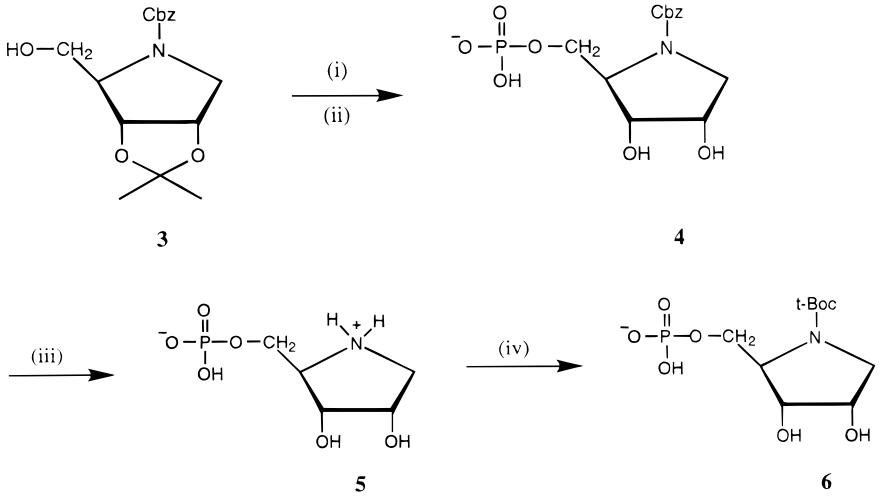
*Poly(ADP-ribose) Glycohydrolase*. PARG was purified from bovine thymus as described previously (*17*). The recombinant PARG−GST fusion protein was produced, isolated, and purified according to the procedure described previously (*18*). Recombinant PARG could be obtained by treatment of the fusion protein with thrombin [2% (w/w)] at 4 °C for 18 h.

*[$^{32}$P]Poly(ADP-ribose)*. Labeled polymer for glycohydrolase assay was synthesized at high specific activity using the procedure described by Keihlbach et al. (*19*) except that core histones were omitted from the incubation. After trichloroacetic acid precipitation, poly(ADP-ribose) was released from protein by treatment with 0.5 M KOH and 50 mM EDTA at 30 °C for 30 min and purified by chromatography on a DHB-Bio Rex 70 column (*20*).

*Enzyme Assay*. Poly(ADP-ribose) glycohydrolase was assayed at 37 °C and pH 7.5 by measuring the release of [$^{32}$P]ADP-ribose from [$^{32}$P]poly(ADP-ribose) as described previously by Menard and Poirier (*21*). The substrate [$^{32}$P]-poly(ADP-ribose) was present at a monomer concentration of 10 μM in ADP-ribose residues. [$^{32}$P]ADP-ribose which was liberated was separated from the polymer by TLC on polyethyleneimine-impregnated cellulose sheets (0.3 M LiCl/0.9 M acetic acid); the spot was excised and quantitated radiometrically.

*ADP-Ribose Polymers*. ADP-ribose polymers of defined size were prepared enzymatically and purified using DHB-Bio Rex 70 affinity chromatography followed by anion exchange HPLC as described earlier (*19*).

Scheme 1[a]




[a] (i) $POCl_3$/water/pyridine (4:2:4) in acetonitrile, 0 °C, 2 h; (ii) $H^+/H_2O$; (iii) $H_2$, Pd on C; (iv) di-*tert*-butyl dicarbonate, triethylamine.

*Photoaffinity Labeling.* Poly(ADP-ribose) glycohydrolase (5 $\mu$g) was incubated either with the photoprobe alone or with the effector followed by the photoprobe in 50 mM Tris-HCl at pH 8.0 (photolysis buffer) in a final volume of 50 $\mu$L at 0 °C for 10 min before photolysis. Photolysis was performed with a hand-held Mineralight lamp [$\lambda_{max}$ of emission of 254 nm, lamp intensity of 4000 $\mu$W/cm$^2$, model UVG-54, UVP (Upland, CA)] at 0 °C for 5 min at a distance of 2 cm. The intensity of the lamp at a distance of 2 cm (measured from the filter panel of the lamp to the top of the 1 mL Eppendorf tube which contained the sample) was 3300 $\mu$W/cm$^2$. After irradiation, protein was precipitated by application of 20 $\mu$L aliquots to 1 in. squares on a Whatman 3 filter paper disk that had been impregnated with 10% (w/v) trichloroacetic acid in ether and air-dried. Unbound ligand was removed by immersing the filter paper disk in aqueous 5% (w/v) trichloroacetic acid and gently rocking for 15 min. After four such washes, the disk was finally washed with anhydrous methanol and air-dried for 15 min. The individual squares were cut out, and radioactivity was quantitated by liquid scintillation counting.

*Gel Electrophoresis.* SDS−PAGE was performed on a running gel containing 7.5% acrylamide in 0.1% SDS (*22*) with an acrylamide:bisacrylamide ratio of 36.5:1 (w/w). The sample solution was prepared by diluting 10 $\mu$L of sample containing 1 $\mu$g of protein with 10 $\mu$L of sample buffer (31.25 mM Tris, 10% $\beta$-mercaptoethanol, 20% glycerol, 4% SDS, and 0.05% bromophenol blue). The sample solution was heated in a boiling water bath for 5 min and applied to the gel. The electrophoresis was run at a constant voltage of 80 V. After the gel was stained with Coomassie Brilliant Blue R-250 [0.1% (w/v) in 40% methanol and 10% acetic acid] and destained in a solution of 40% methanol/10% acetic acid, the gel was dried on a gel dryer at 80 °C under vacuum for 2 h. The dried gel was exposed to BioMax MR-1 film (Sigma) for autoradiography.

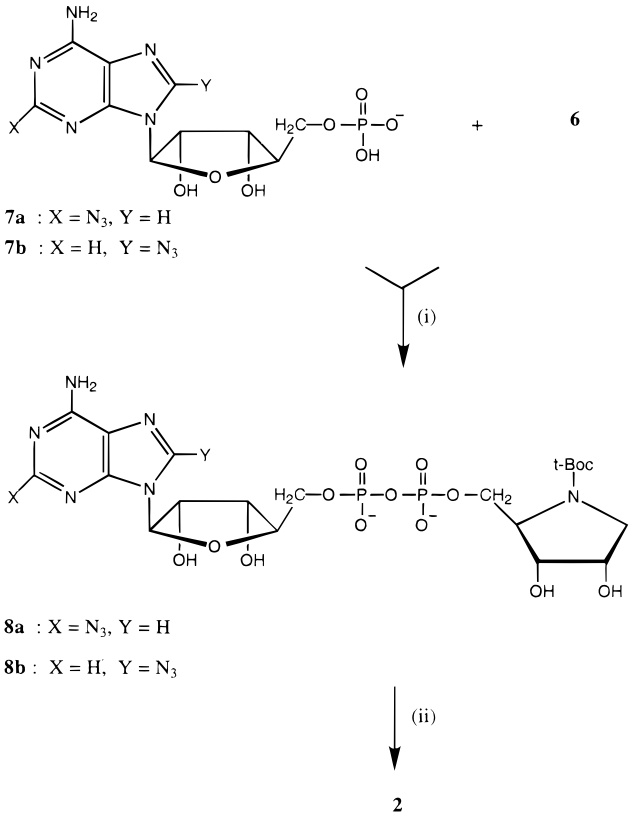
## RESULTS

*Synthesis of Photolablels.* The synthesis of 2-$N_3$-ADP-HPD (**2a**) and 8-$N_3$-ADP-HPD (**2b**) began with the protected pyrrolidine **3** (Scheme 1) which we synthesized from (2*S*)-3,4-dehydroproline according to the procedure of Goli et al. (*9*). Pyrrolidine **3** was phosphorylated using $POCl_3$ in a mixed solvent of water, pyridine, and acetonitrile (*23*). The product **4** was purified by open column chromatography on a reversed phase adsorbent followed by anion exchange chromatography. Since the azide group in the target photoprobe is sensitive to reduction by catalytic hydrogenation, **4** is not suitably protected for the synthesis of **2**. The protecting group of **4** was therefore removed by hydrogenolysis to yield **5**, and the nitrogen was reprotected with the *tert*-butyloxycarbonyl group to yield **6**. The *t*-Boc group of **6** could be removed by acid cleavage, conditions that are compatible with the presence of the 8-azide.

The 2-$N_3$-5′-AMP, **7a**, was prepared in three steps starting from 2-chloroadenosine according to published procedures (*10*, *11*, *24*). The 8-$N_3$-5′-AMP, **7b**, was prepared by selectively phosphorylating commercially available 8-azidoadenosine on the primary 5′-hydroxyl with $POCl_3$ dissolved in trimethyl phosphate (*11*). The coupling of phosphate ester **7a** or **7b** with **6** to produce the protected dinucleotide **8a** or **8b** was performed according to the procedure of Michelson (*25*) (Scheme 2). The trioctylammonium salt of 2-$N_3$-5′-AMP or 8-$N_3$-5′-AMP was activated by reaction with diphenylphosphorochloridate. The resulting mixed anhydride reacted with the anion of **6** in pyridine to produce *t*-Boc-2-$N_3$-ADP-HPD (**8a**) or *t*-Boc-8-$N_3$-ADP-HPD (**8b**). The protected dinucleotide was deprotected on treatment with trifluoroacetic acid to yield 2-$N_3$-ADP-HPD (**2a**) or 8-$N_3$-ADP-HPD (**2b**).

*Chemical and Spectroscopic Properties of the Photolabels.* The photoaffinity labels 2-$N_3$-ADP-HPD and 8-$N_3$-ADP-HPD were obtained as white, amorphous solids following lyophilization. They were stable to fluorescent room light during purification, in aqueous solution and as solids. In aqueous solution at neutral pH, the azide form of **2a** is in equilibrium with tautomeric tetrazole isomers (*24*, *26*). The ultraviolet absorption spectrum of 2-$N_3$-ADP-HPD in aqueous phosphate buffer at pH 7 showed absorption maxima at 310, 271, and 230 nm. The band at 271 nm is attributed to the 2-azide isomer, while the band at 310 nm is characteristic of the tetrazolo isomer. In the case of *t*-Boc-2-$N_3$-ADP-HPD and 2-$N_3$-ADP-HPD, it was possible to partially separate the two isomers that appeared as two distinct peaks

Scheme 2[a]




[a] (i) (1) Trioctylamine, anhydrous methanol; (2) diphenylphosphorochloridate, tributylamine; (ii) trifluoroacetic acid.

Table 1

| compound | TLC[a] $R_f$ | HPLC retention time (min) |
|---|---|---|
| *t*-Boc-2-$N_3$-ADP-HPD, **8a** | 0.54 | 13.8, 15.2 |
| *t*-Boc-8-$N_3$-ADP-HPD, **8b** | 0.59 | 14.3 |
| 2-$N_3$-ADP-HPD, **2a** | 0.37 | 3.3, 6.1 |
| 8-$N_3$-ADP-HPD, **2b** | 0.27 | 2.4 |

[a] TLC (silica gel, 6:3:1 2-propanol/concentrated $NH_4OH$/water).

by reversed-phase HPLC (Table 1). However, when the isolated azide material was examined by HPLC, it was found to re-equilibrate into the tetrazole−azide isomers. The absorption spectra of each of the two peaks corresponding to the tautomers of 2-$N_3$-ADP-HPD were individually determined using a photodiode array detector. The leading peak (3.3 min) exhibited absorption maxima at 310 and 230 nm that were characteristic of the tetrazole isomer, and the second peak (6.1 min) exhibited absorption maxima at 271 and 230 nm that were characteristic of the azide isomer. 8-$N_3$-ADP-HPD did not exhibit a tautomeric equilibrium. The UV absorption spectrum of **2b** in neutral aqueous solution exhibited absorption maxima at 230 and 281 nm ($\epsilon_{281} = 1.4 \times 10^4$ $M^{-1}$ $cm^{-1}$) characteristic of the azido form. The presence of the azide group in **2b** was confirmed by the strong absorption observed at 2159 $cm^{-1}$ in the IR spectrum and by the reduction of the azide to an amine using dithiothreitol (DTT). An aqueous solution of DTT (pH 10) was added to a solution of 8-$N_3$-ADP-HPD in the dark. The UV absorption maximum shifted from 281 to 274 nm. This shift in absorption maximum is consistent with the values reported for conversion of an 8-purinyl azide to the 8-amine (*27*). To demonstrate the characteristic photoreactivity of

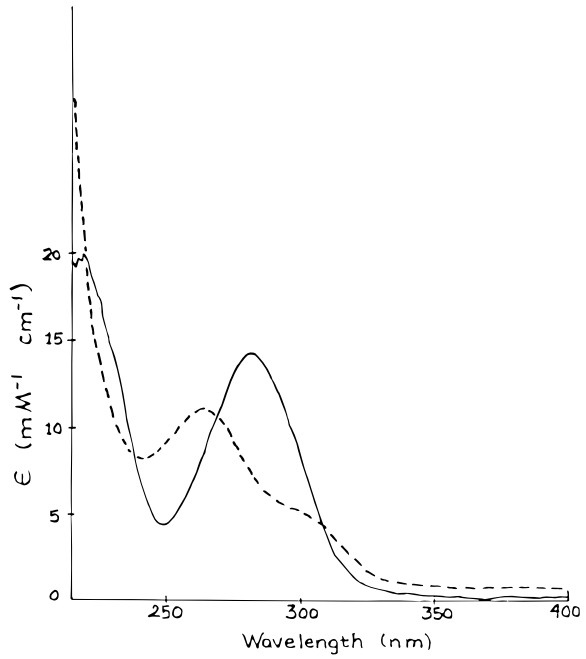



FIGURE 1: Ultraviolet irradiation of 8-azido-ADP-HPD. The 8-$N_3$-ADP-HPD was dissolved in 50 mM potassium phosphate buffer at pH 7.0 and its absorption spectrum recorded (—). The solution was irradiated with a hand-held Mineralight UV lamp (254 nm) until no further changes in the spectrum were observed, at which time the final spectrum was measured (- - -).

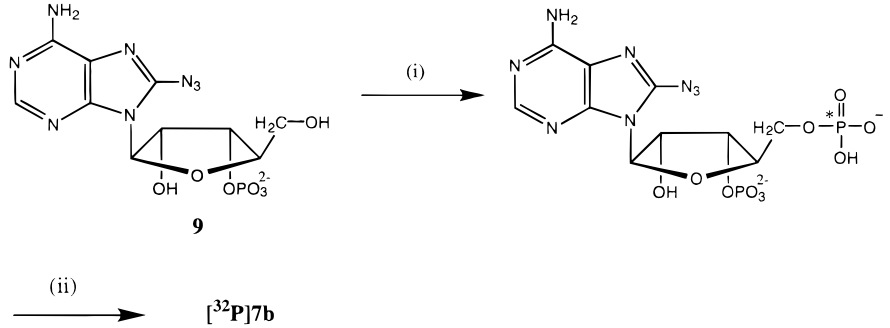
the 8-azido group, a buffered solution of **2b** (50 mM $KH_2PO_4$ at pH 7.0) was irradiated with short-wavelength UV light and the absorption spectrum measured at intervals (Figure 1). The chromophore changed upon irradiation, forming a new chromophore with an absorbance maximum at 260 nm and a long-wavelength shoulder at 300 nm.

*Inhibition of Poly(ADP-ribose) Glycohydrolase*. The ability of 2-$N_3$-ADP-HPD and 8-$N_3$-ADP-HPD to inhibit poly(ADP-ribose) glycohydrolase was determined by adding varying concentrations of inhibitor to assay mixtures and measuring the resulting initial rate of ADP-ribose polymer hydrolysis. The results, presented in Figure 2, show that glycohydrolase activity was inhibited by 50% ($IC_{50}$) at 80 $\mu$M 2-$N_3$-ADP-HPD. The $IC_{50}$ for 8-$N_3$-ADP-HPD was approximately 1 $\mu$M, 80-fold lower than that of the 2-azido isomer and roughly equivalent to that of the parent nucleotide ADP-HPD. 8-$N_3$-ADP-HPD was the superior inhibitor and therefore the photoprobe of choice for photoaffinity labeling studies on poly(ADP-ribose) glycohydrolase.

*Synthesis of [α-$^{32}$P]-8-$N_3$-ADP-HPD*. The radioactive photolabel [α-$^{32}$P]-8-$N_3$-ADP-HPD was prepared by coupling [$^{32}$P]-8-$N_3$-5′-AMP (Scheme 3) and 1-(*tert*-butyloxycarbonyl)-2-[(phosphooxy)methyl]pyrrolidinediol (**6**) according to the method of Hoard and Ott (*28*) (Scheme 4). In this procedure, the phosphate group of **6** was activated by reaction with *N*,*N*′-carbonyldiimidazole to form an imidazolide derivative **10**, which was then reacted with [$^{32}$P]-8-$N_3$-5′-AMP. The protected dinucleotide was treated with trifluoroacetic acid to obtain [α-$^{32}$P]-8-$N_3$-ADP-HPD ([$^{32}$P]**2b**).

*Photoaffinity Labeling*. Irradiation of [α-$^{32}$P]-8-$N_3$-ADP-HPD at low concentrations and an engineered recombinant PARG−GST fusion protein (*18*) with short-wavelength UV light resulted in covalent labeling of the protein (Figure 3A). The covalent incorporation of the photoprobe into a 91 kDa

Scheme 3[a]




[a] (i) [$\gamma$-$^{32}$P]ATP, ATP, T4 polynucleotide kinase, incubation at 37 °C for 18 h; (ii) nuclease P1, incubation at room temperature for 18 h.

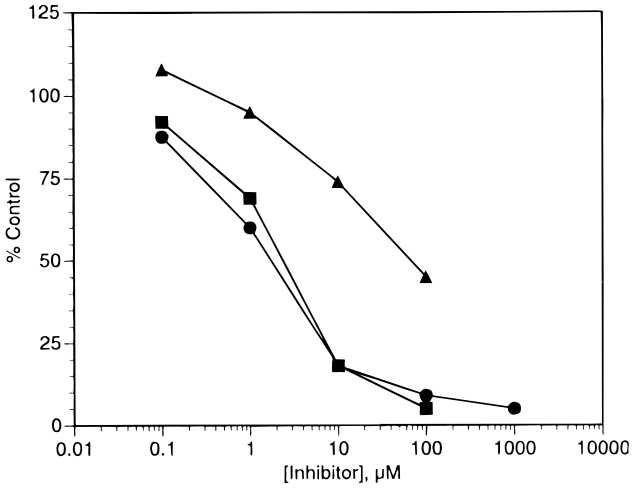
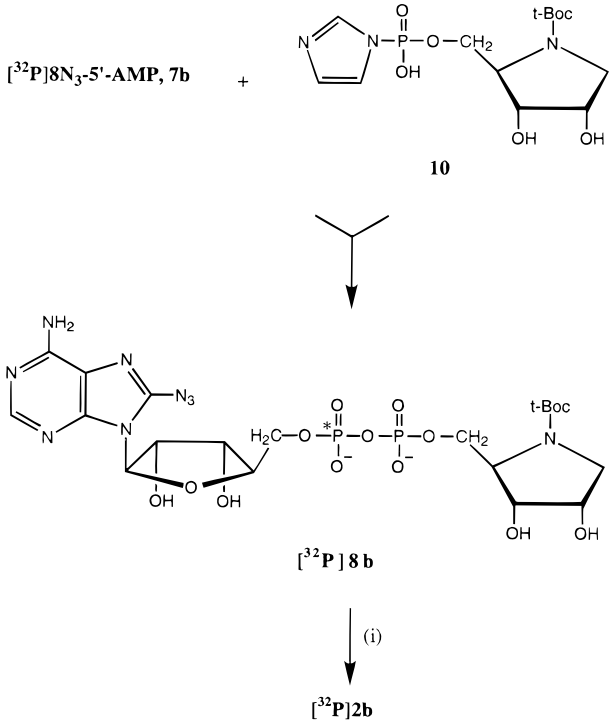



FIGURE 2: Inhibition of poly(ADP-ribose) glycohydrolase by 2- and 8-azido-ADP-HPD and ADP-HPD. Varying amounts of 2-$N_3$-ADP-HPD (▲), 8-$N_3$-ADP-HPD (●), and ADP-HPD (■) were added to the enzyme assay at pH 7.5 containing the substrate [$^{32}$P]-poly(ADP-ribose) at a monomer concentration of 10 $\mu$M. [$^{32}$P]ADP-ribose that formed was separated from [$^{32}$P]poly(ADP-ribose) by TLC on polyethyleneimine cellulose sheets (0.3 M LiCl/0.9 M acetic acid); the spot was cut out and the amount of ADP-ribose determined radiometrically.

protein corrosponding to the PARG−GST fusion protein was observed qualitatively when the photoderivatized samples were subjected to analysis by SDS gel electrophoresis followed by autoradiography. No photoincorporation was observed if the reaction mixture was not irradiated with UV light. The amount of labeling decreased in the presence of 0.2 and 1.0 $\mu$M ADP-HPD, as shown by lighter bands on the autoradiogram. Addition of ADP (1 $\mu$M) did not cause an observable change in the amount of labeling of the 91 kDa band.

The substrate protein in these photolabeling studies was expressed in *Escherichia coli* as a fusion protein in which the 26 kDa glutathione *S*-transferase (GST) was fused to the N terminus of the 65 kDa PARG (*18*). It was therefore important to demonstrate at the outset that photoincorporation occurred exclusively within the PARG domain of the fusion protein and not in the GST domain of the construct. Figure 3B shows the results of thrombin protease digestion of the GST−PARG fusion protein wherein the glutathione *S*-transferase fragment is cleaved off. Upon digestion with thrombin, the radiolabeled 91 kDa band from the GST−PARG fusion protein gave rise to a single 65 kDa radiolabeled band. Irrespective of whether the digestion was carried out before or after photolabeling, the same 65 kDa band

Scheme 4[a]




[a] (i) Trifluoroacetic acid.

representing the PARG domain of the construct was exclusively photolabeled.

The conditions employed for photoincorporation (irradiation for 5 min at 0 °C with 2 cm separating the lamp filter from the top of the microcentrifuge tube) did not affect the activity of the rPARG−GST fusion protein. When the enzyme was irradiated alone and its hydrolytic activity assayed, it retained complete activity (108%), compared to a control where the enzyme was not irradiated. Incubation of the enzyme with 8-$N_3$-ADP-HPD (1 $\mu$M) and irradiation under the same conditions caused a reduction in the enzyme activity of about 54% (data not shown).

Quantitative data for photoincorporation under varying conditions of photolabeling were obtained by the method of acid precipitation as described in Experimental Methods. Upon irradiation of 1 $\mu$M [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD with the fusion protein for various lengths of time, it was observed that the extent of photoincorporation increased, reaching a maximum value after 5 min. In the absence of irradiation, the extent of labeling was reduced to less than 2% of that of the control. When the photolabel was irradiated prior to

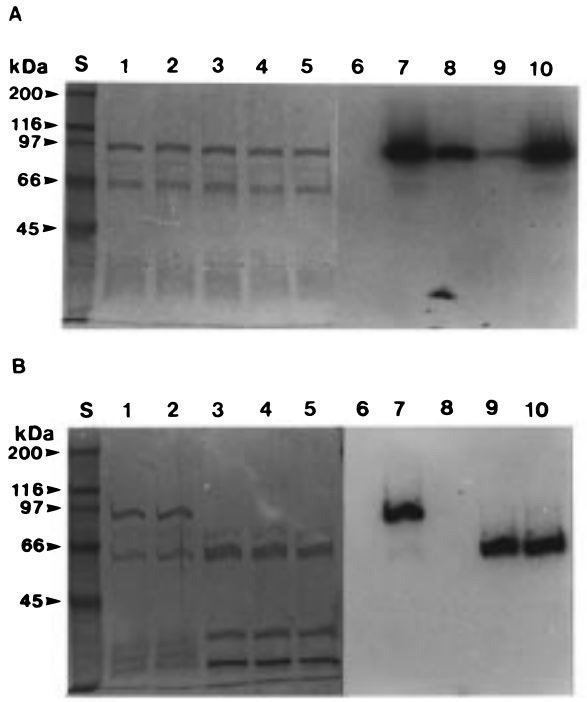



FIGURE 3: SDS−PAGE of photoaffinity labeling of the rPARG−GST fusion protein with [α-$^{32}$P]-8-$N_3$-ADP-HPD (A) and digestion by thrombin protease (B). Lanes 1−5 represent visualization of protein bands by Commassie blue staining and lanes 6−10 by autoradiography. Lane S shows standard molecular mass markers. (A) The rPARG−GST fusion protein (5 μg) was incubated with 1 μM [α-$^{32}$P]-8-$N_3$-ADP-HPD either alone (lanes 1 and 6 or lanes 2 and 7) or in the presence of 0.2 μM ADP-HPD (lanes 3 and 8), 1.0 μM ADP-HPD (lanes 4 and 9), or 1.0 μM ADP (lanes 5 and 10) and irradiated. In lanes 1 and 6, the protein was incubated with the photolabel but not irradiated. (B) The rPARG−GST fusion protein (5 μg) either photolabeled with 1 μM [α-$^{32}$P]-8-$N_3$-ADP-HPD (lanes 2 and 7) or without photolabeling (lanes 1 and 6) was digested with thombin protease (lanes 4 and 9 and 3 and 8, respectively) at 4 °C for 18 h while the mixture was gently shaken. In lanes 5 and 10, the fusion protein was digested with thrombin protease prior to photolabeling.

incubation with the fusion protein, only 7% labeling was achieved. Also, preirradiated photolabel, when incubated with the fusion protein and when the mixture was irradiated again, produced only 3% photoincorporation (Figure 4).

The effect of including low concentrations of various nucleotides—ATP, ADP, AMP, NAD, HPD-P (**5**), and ADP-HPD (**1**)—on the extent of photoincorporation was studied (Figure 4). None of the nucleotides except ADP-HPD exerted any appreciable influence on the extent of photoincorporation. However, in the presence of 1 μM ADP-HPD, the extent of photoincorporation decreased to a value of about 11% of that of the control. The presence of divalent metal ions and the chelating agent had varied effects on photoincorporation. The divalent metal ions $Ca^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ present at a concentration of 10 mM did not change the extent of photoincorporation. In the presence of 10 mM EDTA, the extent of photoincorporation was 2-fold greater than that of the control.

In Figure 5A, we report the result of the effect of varying the initial concentration of photoprobe on the amount of nucleotide covalently attached to the protein. When 0.1 nmol of the fusion protein was photolabeled with increasing concentrations of [α-$^{32}$P]-8-$N_3$-ADP-HPD (0−2.0 μM), the extent of covalent labeling increased but reached a maximum

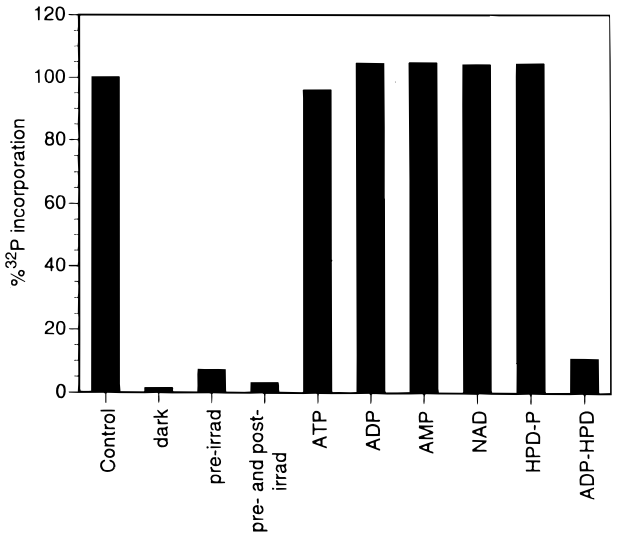



FIGURE 4: Protection of [α-$^{32}$P]-8-$N_3$-ADP-HPD photoinsertion into the rPARG−GST fusion protein under various conditions. Fusion protein (0.1 nmol) in photolysis buffer (final volume of 50 μL) at 0 °C containing 1 μM [α-$^{32}$P]-8-$N_3$-ADP-HPD was treated as follows: control, fusion protein and photoprobe incubated for 10 min, followed by irradiation for 5 min; dark, fusion protein and photoprobe incubated for 10 min, followed by continued incubation for 5 min in the dark; pre-irrad, photoprobe irradiated for 5 min in photolysis buffer, followed immediately by addition of fusion protein and incubation for 10 min; pre- and post-irrad, photoprobe irradiated for 5 min in photolysis buffer, followed immediately by addition of fusion protein, incubation for 10 min, and irradiation of the mix for 5 min; or, fusion protein incubated with the designated nucleotide (1 μM) in photolysis buffer for 10 min at 0 °C, followed by addition of [α-$^{32}$P]-8-$N_3$-ADP-HPD (1 μM), incubation for an additional 10 min at 0 °C, and irradiation for 5 min. $^{32}$P incorporation was measured by precipitation of a 20 μL aliquot on acid-treated paper followed by washing to remove unbound nucleotide and quantitation by liquid scintillation counting as described in Experimental Methods.

value at about 1.0 μM, with an apparent $K_d$ of approximately 0.3 μM. At a photoprobe concentration of 1.0 μM, the stoichiometry of photolabeling was 0.17 mol/mol of fusion protein.

When the fusion protein was labeled with 1 μM photoprobe in the presence of increasing concentrations of ADP-HPD, covalent photolabeling was observed to decrease significantly. Approximately 90% of the labeling was inhibited in the presence of 0.8 μM ADP-HPD (Figure 5B). The half-maximal protection of labeling was observed at 0.2 μM ADP-HPD.

The effect of addition of ADP-ribose polymers of varying size on photoincorporation was studied (Figure 6). When 0.15 nmol of the protein was irradiated with 1 μM [α-$^{32}$P]-8-$N_3$-ADP-HPD in the presence of 1 μM ADP-ribose polymer (5-mer and 15-mer), only 13−14% photoincorporation was obtained. In contrast, inclusion of 1 μM 30−50-mer resulted in an extent of photoincorporation of 129% compared to that of the control that did not contain any ADP-ribose polymer. At 10 μM, the 30−50-mer gave an extent of photoincorporation of 103%.

## DISCUSSION

Poly(ADP-ribose) glycohydrolase is the enzyme primarily responsible for degradation of ADP-ribose polymers. Since this metabolism has been shown to be involved in many

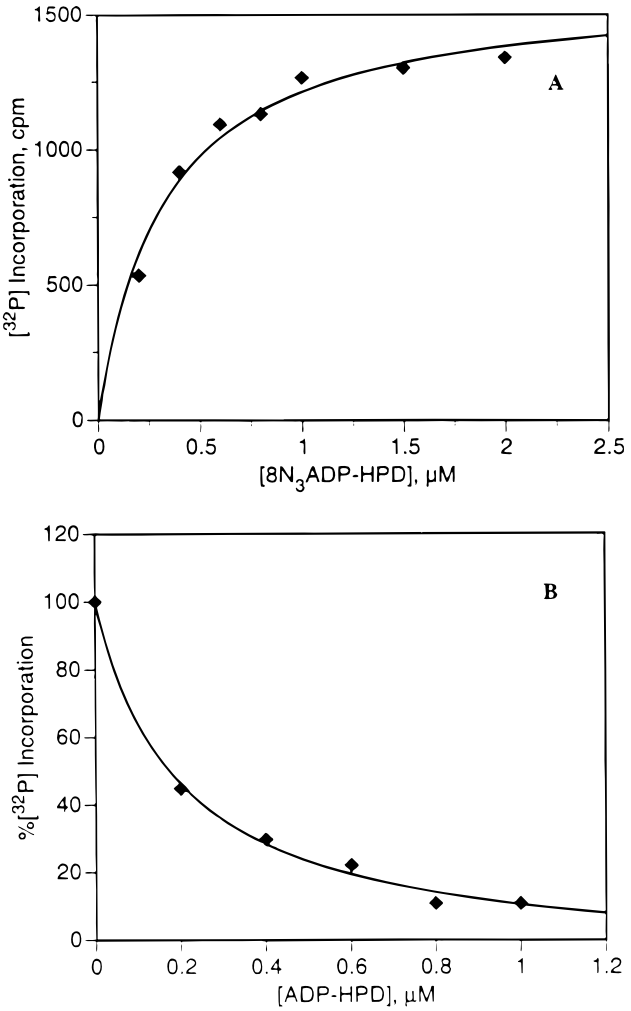



FIGURE 5: Effect of the concentration of **[**α-$^{32}$P]-8-$N_3$-ADP-HPD (A) or ADP-HPD (B) on photoincorporation of [α-$^{32}$P]-8-$N_3$-ADP-HPD into the rPARG−GST fusion protein. (A) Fusion protein (0.1 nmol) in 100 $\mu$L of photolysis buffer was incubated with varying concentrations of [α-$^{32}$P]-8-$N_3$-ADP-HPD (0−2.0 $\mu$M) for 10 min at 0 °C and photolyzed for 5 min. $^{32}$P incorporation was determined as acid-precipitable radioactivity. Data were fit to the equation cpm = cpm$_\infty$[**2b**]/($K_d$ + [**2b**]) by using a nonlinear regression analysis, and the values for cpm$_\infty$ and $K_d$ were computed. The solid line represents the theoretical line calculated using the empirically determined values of cpm$_\infty$ and $K_d$ (1607 and 0.32 $\mu$M, respectively). (B) Fusion protein (0.1 nmol) was incubated in photolysis buffer with varying concentrations of ADP-HPD (0−1.0 $\mu$M) for 10 min at 0 °C. [α-$^{32}$P]-8-$N_3$-ADP-HPD (1 $\mu$M) was added and the solution incubated for an additional 10 min. The mixture was photolyzed for 5 min at 0 °C. $^{32}$P incorporation was determined as acid-precipitable radioactivity. Data were fit to the equation cpm = 100% − %$_\infty$[**2b**]/($K_d$ + [**2b**]) by using a nonlinear regression analysis, and the values for %$_\infty$ and $K_d$ were computed. The solid line represents the theoretical line calculated using the empirically determined values of %$_\infty$ and $K_d$ (107 and 0.2 $\mu$M, respectively).

cellular processes and particularly in the recovery of dividing cells from DNA damage, this enzyme is being actively studied. Adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD, **1**) has been shown to be a potent and specific inhibitor of poly(ADP-ribose) glycohydrolase (*7*). Kinetic studies using high-molecular weight, branched ADP-ribose polymer as a substrate showed ADP-HPD to be a partial, noncompetitive inhibitor of poly(ADP-ribose) glycohydrolase ($K_{ii}$ = 52 ± 10 nM and $K_{is}$ = 80 ± 20 nM) (*29*). Photoactive analogues of ADP-HPD can be used to obtain information about the binding of ADP-HPD to the

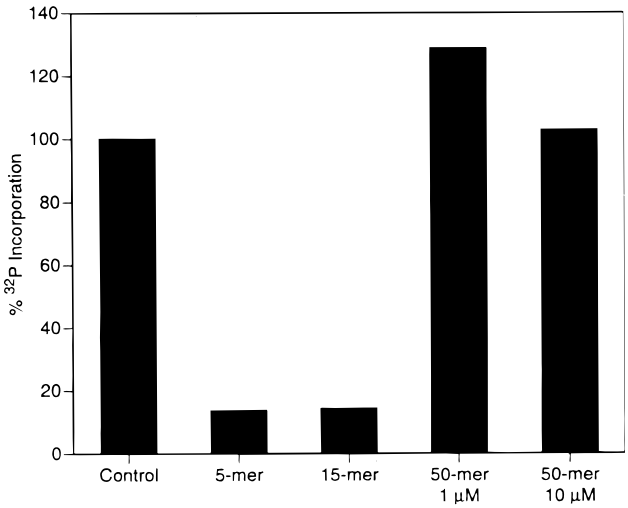



FIGURE 6: Effect of ADP-ribose polymers of varying size on photoincorporation of **[**α-$^{32}$P]-8-$N_3$-ADP-HPD into rPARG. The rPARG (0.15 nmol) in phosphate-buffered saline was incubated with 1 $\mu$M [α-$^{32}$P]-8-$N_3$-ADP-HPD for 10 min at 0 °C. ADP-ribose polymers of varying sizes (5-, 15-, and 50-mer) were added to a concentration of 1 $\mu$M of the polymer. The reaction mixture was immediately irradiated for 5 min at 0 °C. $^{32}$P incorporation was determined as acid-precipitable radioactivity. A control experiment that did not contain any polymer was designated as 100% $^{32}$P incorporation.

glycohydrolase and to identify the peptide fragments and the amino acid residues that are involved in inhibitor binding. This information is expected to provide insight into the structure, mechanism, and regulation of the enzyme.

Two isomeric azidoadenosine analogues of ADP-HPD, 2-$N_3$-ADP-HPD and 8-$N_3$-ADP-HPD, were synthesized and characterized. Inhibition of glycohydrolase activity by the two photoactive analogues of ADP-HPD showed 8-$N_3$-ADP-HPD to inhibit at a concentration of 1 $\mu$M, 80-fold lower than that of 2-$N_3$-ADP-HPD. This suggested that substitution at the 8-position of ADP-HPD was tolerated much better by the enzyme than substitution at the 2-position. Since 8-substituted adenosine nucleotides prefer the syn conformation (*24*, *30*), the superior binding of **2b** might mean that the enzyme binds ADP-HPD in the syn conformation. Since 8-$N_3$-ADP-HPD was the superior inhibitor, it was selected for further development as a photoaffinity label for poly(ADP-ribose) glycohydrolase, and the radioactive analogue [α-$^{32}$P]-8-$N_3$-ADP-HPD was synthesized.

When rPARG was irradiated with low concentrations of [α-$^{32}$P]-8-$N_3$-ADP-HPD (1 $\mu$M), nucleotide was covalently incorporated into the protein. The covalent derivatization required UV light and that enzyme and the azide-containing photolabel both be present at the time of irradiation. Irradiation of the photoprobe to activate the photoactive azide by photolysis followed by immediate addition of the enzyme did not lead to covalent labeling, an indication of the absence of any long-lived chemically reactive intermediate that could covalently modify poly(ADP-ribose) glycohydrolase. Also, prephotolyzed photoprobe was shown to be incapable of causing significant photolabeling. This indicated that the labeling process was a result of a photochemical production of a short-lived intermediate directly from the azide (i.e., a nitrene). The enzyme activity was fully retained when the enzyme was irradiated in the absence of the photolabel, indicating that the UV light under the conditions employed

for photoincorporation did not cause any damage to the enzyme.

Nucleotides such as ATP, ADP, AMP, and NAD present at a concentration of 1 $\mu$M did not compete with [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD for binding to the enzyme as shown by the failure to cause any significant change in the extent of photolabeling. The exception was ADP-HPD, which at 1 $\mu$M reduced the extent of photoincorporation by 90%. Thus, ADP-HPD and 8-$N_3$-ADP-HPD must compete for the same binding site on the enzyme. It also suggests that, if PARG has any regulatory nucleotide binding sites, ADP-HPD does not inhibit the enzyme by binding at those sites.

Saturation of photoincorporation at low concentration of the photoaffinity label and photoprotection by the parent ligand are the two most important criteria for demonstrating specific photolabeling. The saturation of photoincorporation with increasing concentrations of the photolabel (Figure 5A) and saturable photoprotection by ADP-HPD (Figure 5B) support specific, covalent photoincorporation. The photoinsertion with 8-$N_3$-ADP-HPD was saturated with an apparent $K_d$ of 0.3 $\mu$M. This is in agreement with the $IC_{50}$ value of 1.4 $\mu$M obtained from a competitive enzyme inhibition assay (Figure 2). Likewise, the $K_{d(app)}$ for the photoprotective effect of ADP-HPD was calculated to be 0.2 $\mu$M from the photoprotection experiment and was similar to an $IC_{50}$ of 0.12−2 $\mu$M determined previously (*7*) and in Figure 2. The similarity between the $K_{d(app)}$ for photoprotection by ADP-HPD and the $IC_{50}$ for ADP-HPD indicates that the photolabeling was occurring at the ADP-HPD binding site. Thus, [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD will be useful in the identification of the peptide fragments and the amino acid residues that constitute the ADP-HPD binding site of poly(ADP-ribose) glycohydrolase.

It was interesting to determine if ADP-ribose polymers, which are the natural substrate of poly(ADP-ribose) glycohydrolase, would compete with the photolabel for binding to the enzyme. Unbranched oligomers containing 5 (5-mer) and 15 ADP-ribose units (15-mer) as well as high-molecular weight, branched polymers containing 30−50 ADP-ribose units (50-mer) were tested. While the 5-mer and the 15-mer reduced the extent of photoderivatization by 85%, the 50-mer was found to actually increase the extent of photoincorporation to about 129% at 1 $\mu$M. These results indicate that the low-molecular weight, unbranched polymers were competing with [$\alpha$-$^{32}$P]-8-$N_3$-ADP-HPD for a common binding site. A similar amount of photoprotection was obtained with ADP-HPD (Figure 4) at 1 $\mu$M. Thus, ADP-HPD is binding to a site on PARG where low-molecular weight, unbranched polymers bind. The high-molecular weight, branched ADP-ribose polymers bind at a site different from that of the short, linear polymers and ADP-HPD. The mechanism of inhibition of ADP-HPD was previously studied using an enzyme inhibition assay (*29*) that employed high-molecular weight, branched ADP-ribose polymer as the substrate and ADP-HPD as the inhibitor. It was found that ADP-HPD behaved as a partial, noncompetitive inhibitor toward the high-molecular weight, branched polymer substrate. This mechanism required the formation of a ternary complex containing enzyme, inhibitor, and high-molecular weight polymer and that this ternary complex be capable of forming product. The existence of this ternary complex is consistent with the observation that high-molecular weight, branched polymer and 8-$N_3$-ADP-HPD fail to compete for a binding site. Since short unbranched polymers are known to be hydrolyzed by PARG to ADP-ribose, our observation of competition between 8-$N_3$-ADP-HPD and short oligomers for a common binding site suggests that ADP-HPD may be found to be a competitive inhibitor for the hydrolysis of low-molecular weight, unbranched polymers. The results of the photolabeling experiment in the presence of varying sizes of ADP-ribose polymers suggest that there are two separate binding sites on poly(ADP-ribose) glycohydrolase, one for low-molecular weight, linear polymers and one for high-molecular weight, branched polymers.

The hydrolysis of high-molecular weight, branched poly(ADP-ribose) is known to be complex. Hydrolysis of high-molecular weight, branched substrate is biphasic (*31*), with an initial rapid processive hydrolysis proceeding until about 50% of the polymer is consumed followed by a slow distributive hydrolysis into ADP-ribose monomers. Poly(ADP-ribose) glycohydrolase catalyzes exoglycosidic as well as endoglycosidic cleavage of substrate (*32*−*34*). The enzyme also processes branched polymers. These different modes of activity could accommodate either multiple catalytic sites or allosteric regulation by short oligomers.

The existence of dual ADP-ribose polymer binding sites has not been reported previously. Indeed, we do not yet know whether the ADP-HPD binding site is catalytically active or if it is purely regulatory. The release and binding of ADP-ribose oligomers during ADP-ribose polymer turnover in vivo may constitute a signaling mechanism that may contribute to cell survival in the aftermath of DNA damage. Further investigation of the dual binding sites of poly(ADP-ribose) glycohydrolase will be necessary to provide more information for understanding the complex nature of the degradation of poly(ADP-ribose), its regulation, and its role in the DNA repair process.

## ACKNOWLEDGMENT

We thank Donna Coyle for her technical assistance in various aspects of this work.

## REFERENCES

1. Ueda, K., and Hayashi, O. (1985) *Annu. Rev. Biochem. 54*, 73−100.
2. De Murcia, G., Huletsky, A., and Poirier, G. G. (1988) *Biochem. Cell Biol. 66*, 626−635.
3. Ferro, A. M., and Oppenheimer, N. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 809−813.
4. Miwa, M., Ishihari, M., Takishima, S., Takasuka, N., Maeda, M., Yamaizuma, Z., Sugimura, T., Yokoyama, S., and Miyazawa, T. (1981) *J. Biol. Chem. 256*, 2916−2921.
5. Jacobson, E. L., Smith, J. Y., Wielckens, K., Hilz, H., and Jacobson, M. K. (1985) *Carcinogenesis 6*, 715−718.
6. Juares-Salinas, H., Duran-Toues, G., and Jacobson, M. K. (1984) *Biochem. Biophys. Res. Commun. 122*, 1381−1388.
7. Slama, J. T., Aboul-Ela, N., Goli, D. M., Cheeseman, B. V., Simmons, A. M., and Jacobson, M. K. (1995) *J. Med. Chem. 38*, 389−393.
8. Haley, B. E. (1991) *Methods Enzymol. 200*, 477−487.
9. Goli, D. M., Cheeseman, B. V., Lodaya, R. M., Hassan, M. E., and Slama, J. T. (1994) *Carbohydr. Res. 259*, 219−241.
10. Schaffer, J. H., and Thomas, J. H. (1958) *J. Am. Chem. Soc. 80*, 3738−3742.
11. Yoshikawa, M., Kato, T., and Takenishi, T. (1969) *Bull. Chem. Soc. Jpn. 42*, 3505−3508.

12. Wielckens, K., Bredehorst, R., Adamietz, P., and Hiltz, H. (1981) *Eur. J. Biochem. 117*, 69−74.
13. Ikehara, M., and Uesugi, S. (1969) *Chem. Pharm. Bull. 17* (2), 348−354.
14. Czarnecki, J. J., Geahlen, R., and Haley, B. (1979) *Methods Enzymol. 56*, 642−653.
15. Freist, W., and Cramer, F. (1978) in *Nucleic Acid Chemistry, part two* (Townsend, L. B., and Tipson, R. S., Eds.) pp 827−836, Wiley, New York.
16. Walseth, T. F., Aarhus, R., Kerr, J. A., and Lee, H. C. (1993) *J. Biol. Chem. 268*, 26686−26691.
17. Thomassin, H., Jacobson, M. K., Guay, J., Verreault, A., Aboul-Ela, N., Menard, L., and Poirier, G. G. (1990) *Nucleic Acids Res. 18*, 4691−4694.
18. Lin, W., Amé, J.-C., Aboul-Ela, N., Jacobson, E. L., and Jacobson, M. K. (1997) *J. Biol. Chem. 272*, 11895−11901.
19. Kiehlbauch, C. C., Aboul-Ela, N., Jacobson, E. L., Ringer, D. P., and Jacobson, M. K. (1993) *Anal. Biochem. 208*, 26−34.
20. Aboul-Ela, N., Jacobson, E. L., and Jacobson, M. K. (1988) *Anal. Biochem. 174*, 239−250.
21. Menard, L., and Poirier, G. G. (1987) *Biochem. Cell Biol. 65*, 668−673.
22. Laemmli, U. (1970) *Nature 227*, 680−685.
23. Sowa, T., and Ouchi, S. (1975) *Bull. Chem. Soc. Jpn. 48*, 2084−2090.
24. Kim, H., and Haley, B. E. (1990) *J. Biol. Chem. 265*, 3636−3641.
25. Michelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1−13.
26. Czarnecki, J. J. (1984) *Biochim. Biophys. Acta 800*, 41−51.
27. Holmes, R. E., and Robins, R. K. (1965) *J. Am. Chem. Soc. 87*, 1772−1776.
28. Hoard, D. E., and Ott, D. E. (1965) *J. Am. Chem. Soc. 87*, 1785−1788.
29. Slama, J. T., Aboul-Ela, N., and Jacobson, M. K. (1995) *J. Med. Chem. 38*, 4332−4336.
30. Sarma, R. H., Lee, C.-H., Evans, F. E., Yathindra, N., and Sundaralingam, M. (1974) *J. Am. Chem. Soc. 96*, 7337−7348.
31. Hatakeyama, K., Nemoto, Y., Ueda, K., and Hayaishi, O. (1986) *J. Biol. Chem. 261*, 14902−14911.
32. Ikejima, M., and Gill, D. M. (1988) *J. Biol. Chem. 263*, 11037−11040.
33. Brochu, G., Duchaine, C., Thibeault, L., Lagueux, J., Shah, G. M., and Poirier, G. G. (1994) *Biochim. Biophys. Acta 1219*, 342−350.
34. Braun, S. A., Panzeter, P. L., Collinge, M. A., and Althaus, F. R. (1994) *Eur. J. Biochem. 220*, 369−375.

BI9730386